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2. Zarinan et al., Human Reproduction. 2001 august, vol. 16, No. 88, pp. 1611-1618.
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Receptor Binding Activity and In Vitro Biological Activity of the Human FSH Charge Isoforms as Disclosed by Heterologous and Homologous Assay Systems

Implications for the Structure–Function Relationship of the FSH Variants

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Follicle-stimulating hormone (FSH) is produced and secreted in multiple molecular forms. These isoforms differ in their oligosaccharide structures, which determine the particular behavior of a given variant in in vitro and in vivo systems. Employing heterologous cell assay systems, this and other laboratories have shown that highly sialylated human FSH variants exhibit lower receptor binding/immunoactivity as well as in vitro bioactivity/inmunoactivity relationships than their less sialylated counterparts. It is not known, however, whether this characteristic behavior of the FSH isoforms is reproduced by homologous assay systems, in which unique variants of the receptor are presumptively expressed. To gain further insights into the structure–activity relationship of the various FSH isoforms, we analyzed the capacity of nine charge isoforms obtained after high-resolution chromatofocusing (pH window, 7.10 to <3.80) of anterior pituitary glycoprotein extracts to bind and activate their cognate receptor expressed by naturally occurring heterologous cell systems (rat granulosa cells and seminiferous tubule homogenates) as well as by human embryonic kidney-derived 293 (HEK-293) cells transfected with the human FSH (FSH-R) receptor cDNA. In both (heterologous and homologous) receptor assay systems, the isoforms displaced ^{125}I-labeled FSH from the receptor in a dose-response manner; however, whereas in the heterologous systems, the receptor binding activity varied according to the elution pH value/sialic content of the isoforms, with the less acidic variants exhibiting higher receptor binding activity ($r = 0.851$ and 0.495 [$p < 0.01$ and $p < 0.05$] for the granulosa cell and testicular homogenate receptor assay systems, respectively) than the more acidic/sialylated analogs, in the homologous assay, this relationship was practically absent ($r = 0.372$, p N.S.). The capacity of the isoforms to induce androgen aromatization by rat granulosa cells followed the same trend shown by its corresponding receptor assay system ($r = 0.864$, $p < 0.01$). Interestingly and in contrast to the results observed in the homologous receptor binding assay, the ability of the isoforms to induce cAMP production by HEK-293 cells varied according to their elution pH value, with the more sialylated isoforms exhibiting lower potency than their less acidic counterparts ($r = 0.852$, $p < 0.01$). The results yielded by the heterologous assays suggest that the different potency of the isoforms to elicit a biological effect in a naturally occurring receptor system depends primarily on the particular affinity of the receptor molecule for each isoform. The existence of a clear dissociation between receptor binding and signal transduction in the homologous system indicate that this later function is rather related to the different ability of the FSH glycosylation variants to induce and/or stabilize distinct receptor conformations that may permit preferential or different degrees of activation/inhibition of a given signal transduction pathway. Thus, the human FSH receptor-transducer system apparently possesses sufficient versatility to respond in a different manner to glycosylation-dependent diverse FSH signals.

Key Words: Follicle-stimulating hormone; follicle-stimulating iso-hormones; FSH receptor.

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Introduction

Follicle-stimulating hormone (FSH) is one of the major hormones produced by the anterior pituitary gland. Like

other members of the glycoprotein hormone family (which includes also luteinizing hormone [LH], chorionic gonadotropin [CG], and thyroid-stimulating hormone [TSH]), FSH is a heterodimer composed of two glycosylated subunits, α and β *(1,2)*. Oligosaccharide structures on FSH, which play a key role in determining the biological attributes of the hormone, are highly variable and constitute the main chemical basis for isoform formation *(3–5)*. Owing to differences in their physicochemical properties, these glycosylation variants of FSH can be easily isolated by a number of biochemical methods, including charge-based techniques *(5)*.

Multiple FSH charge isoforms have been isolated from anterior pituitary (AP) extracts, serum, and urine of several animal species, including humans *(6–11)*. Although in both unpurified AP extracts and in serum, the full spectrum of FSH isoforms includes isoforms with basic and strongly acidic pH values, the major proportion of isoforms, and the bulk of FSH immunoactivity are usually detected at pH values <5.0 *(5)*. In some experimental animals and in humans, the production and secretion of less acidic FSH isoforms significantly increase during the preovulatory phase of the ovarian cycle as well as during pubertal development, which indicates that the synthesis of the various isoforms is hormonally regulated *(8,9,12–15)*. Previous studies have shown that the more acidic/sialylated variants exhibit longer plasma half-life and higher in vivo biological potency, but lower in vitro biopotency than the less acidic counterparts *(9,16–20)*. However, recent studies from our laboratory showed that a recombinant DNA-derived FSH preparation (which exhibits less sialylated oligosaccaride structures and thence an accelerated plasma clearance rate) acutely induced rat ovarian tissue-type plasminogen activator protein and mRNA synthesis with the same potency, but a different kinetics than a predominantly acidic long-lived FSH analog, with the less sialylated preparation triggering both events earlier than its more acidic counterpart *(21)*. Although the results of this and other previously reported studies *(22,23)* clearly indicate that effectiveness of a given FSH variant to induce a net biological effect depends not only on the circulatory half-life, but also on the ability of the variant to bind and activate its cognate receptor, recent studies have suggested that membrane-expressed variants of the gonadotropin receptor may be additionally involved in hormone action by coupling the ligand–receptor complex to either stimulatory or inhibitory signal transduction pathways *(24–26)*.

To gain further insights on the relationship between receptor binding and signal transduction of the naturally occurring FSH variants, we analyzed the capacity of different human pituitary FSH charge isoforms to bind the receptor, and induce a biological response in vitro employing both heterologous and homologous cell systems, which presumptively express either a variable or a unique FSH receptor (FSH-R) population.

Results and Discussion

FSH charge isoforms were isolated by chromatofocusing, a procedure that separates the hormone variants on the basis of charge, which in turn is mainly determined by the amount of sialylated and, to a lesser extent, sulfated Asn-linked oligosaccharides incorporated into the peptide backbone of the hormone *(27)*. The profile of pH distribution of immunoreactive FSH after high-resolving chromatofocusing of an anterior pituitary glycoprotein extract is shown in Fig. 1. Fractions containing the highest concentrations of immunoactive FSH (as assessed by radioimmunoassay [RIA]) within a single peak or closely neighboring peaks were pooled in seven groups with median elution pH values ranging from 6.32 to 3.93, concentrated, and requantified by RIA as described in Materials and Methods. In addition, FSH-containing fractions corresponding to those proteins that passed through the column unrestricted (pH > 7.10) as well as those recovered after the addition of 1 *M* NaCl to the eluent buffer (pH < 3.8) (Fig. 1; isoforms I and IX, respectively) were also separately pooled and assayed; for the correlation analysis, median pH values of 7.30 and 3.0 were arbitrarily assigned to these FSH isoforms recovered at each end of the pH window. Simultaneous curve fitting of the dose–response curves obtained in the RIA of the isoforms revealed no significant differences among the slopes generated by the FSH standards (LER-907 and FSH-I-SIAFP-1) and the several pools of isoforms fractionated by chromatofocusing (not shown). Based on the results of this quantitative procedure, the doses of FSH isoforms to be tested for receptor binding activity (RBA) and in vitro bioactivity (BA) were accordingly defined.

To examine the RBA of the several human FSH charge variants, each isoform concentrate was quantified by three different radio receptor assay (RRA) systems that employ either rat granulosa cells, rat seminiferous tubule homogenates, or human embryonic kidney (HEK) 293 cells expressing the recombinant human FSH-R as the source of receptors. The competitive displacement curves of ^{125}I-labeled FSH by the nine isoform pools and the hFSH-I-SIAFP standard as well as the relationship between the RRA/immunoassay (RRA/I) activity ratio and the elution pH value of the isoforms are shown in Fig. 2. In each RRA, all isoforms displaced the radiolabeled FSH tracer in a dose-dependent manner and in a similar fashion than the highly purified human FSH-I-SIAFP-1 preparation. In both heterologous RRA systems, the RBA of the intrapituitary FSH isoforms (expressed as the RRA/I FSH activity ratio) decreased as the elution pH of the corresponding isoform declined (r = 0.851, $p < 0.01$, and $r = 0.495$, $p < 0.05$, for the granulosa cells and testicular homogenate receptor systems, respectively), with the exception of isoform I (pH 7.30) in the testicular homogenate assay, in which the variant exhibited an RBA similar to that presented by the most acidic FSH analog. In contrast, the use of human cells expressing the

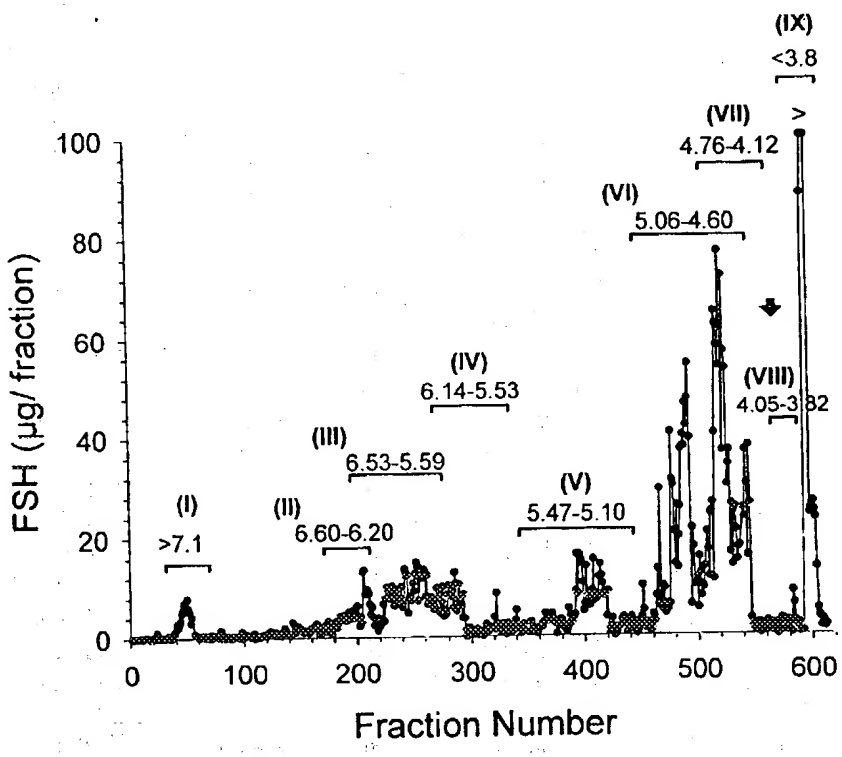

Fig. 1. Representative profile of pH distribution of immunoactive FSH after high-resolution chromatofocusing of an anterior pituitary glycoprotein extract. The elution pH values of the various isoforms (I–IX) are indicated. The arrow marks the addition of 1 *M* NaCl to the chromatofocusing column.

recombinant receptor disclosed a complete absence of differences among the several FSH variants to bind the receptor; as a consequence, the correlation coefficient between the RBA and the elution pH value of the isoforms ($r = 0.372$) did not reach statistical significance.

The ability of the FSH isoforms to compete with radiolabeled FSH for binding to the FSH-R was measured applying the Cheng–Prusoff equation, and the K_d yielded by the saturation studies performed on each receptor binding assay system ($K_d = 3.3 \pm 0.3$, 3.9 ± 0.2, and 2.2 ± 0.2 n*M* for the granulosa cell, seminiferous tubule homogenate, and HEK-293 cell RRA systems, respectively). Compared with the more acidic/sialylated FSH isoforms, the less acidic variants exhibited an increased affinity for the rat FSH receptor as reflected by the lower inhibition constants (K_i) of isoforms with pH values 7.30–6.08 (K_i from 0.14 ± 0.02 to 0.28 ± 0.01 n*M*) in the granulosa cells RRA and isoforms with pH 7.30–5.71 (K_i from 0.55 ± 0.01 to 0.85 ± 0.3 n*M*) in the testicular homogenate RRA system (Fig. 3). In both heterologous RRA systems, there was a significant inverse relationship between the median elution pH value of the isoforms and their corresponding K_i values ($r = -0.822$ and -0.828 for the granulosa cells and the testicular homogenate RRAs). The affinity of the FSH isoforms for the human FSH-R was very similar one from another, with K_i values ranging from 1.25 ± 0.09 to 1.60 ± 0.5; as expected, their K_is and elution pH values were unrelated ($r = -0.563$, *P* NS).

The findings derived from the heterologous RRA systems contrast with previous data where purified human FSH charge isoforms of increased sialic acid yielded the highest specific activities in a heterologous RRA system (calf testis membranes) *(10,28)*. A possible explanation for this apparent discrepancy may be related to differences in the methods used for protein mass determination employed, i.e., amino acid analysis *(10,28)* vs immunoactivity (present study). Although it has been argued that differences in antibody recognition or epitope specificity among the different immunoassay systems may preclude a reliable estimation of the amount of FSH present in a given sample *(28,29)*, it is interesting to note that in a previous study we demonstrated that these potential discrepancies among different (polyclonal, monoclonal, and polyclonal/monoclonal) quantitative immunoassays are rather slight and do not significantly alter the calculated in vitro bioactivity: immunoactivity relationship of the human FSH variants *(30)*. More likely, these conflicting results may be due to the fact that these investigators only included in the analysis those isoforms recovered within a relatively narrow p*I* range (3.50–5.29 vs <3.8 to >7.1 in the present study) *(10,28)*, in which we did not detect differences among the RBA of the isoforms as disclosed by the rat testicular homogenate assay system (*see* Fig. 2B, inset and Fig. 3B).

Differences between the RBA of the isoforms in heterologous and homologous receptor systems may be due to a higher efficiency of the less sialylated FSH variants to dis-

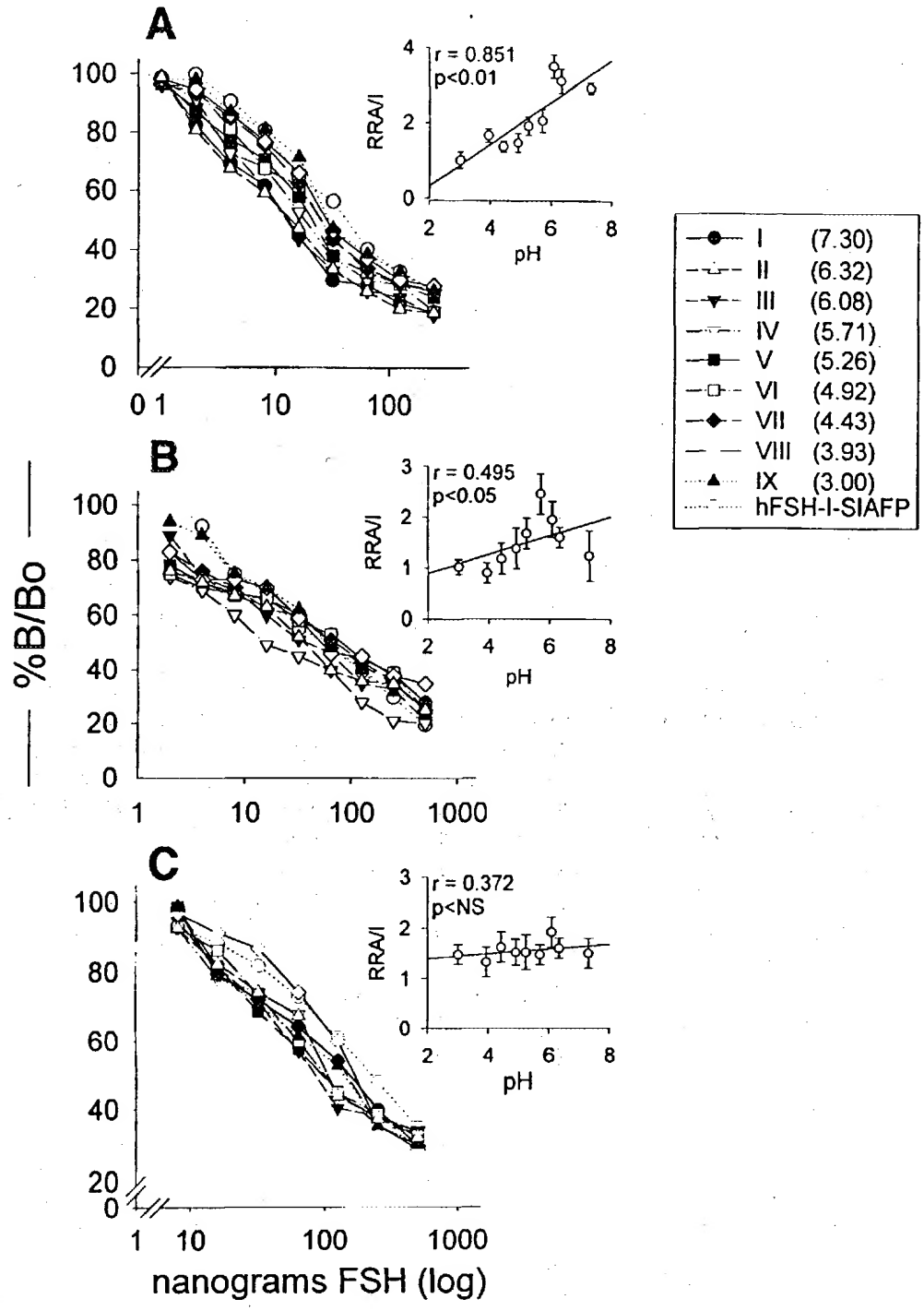

Fig. 2. Representative competitive displacement curves of ^{125}I-labeled FSH by isoforms I–IX and FSH-I-SIAFP-1 in the granulosa cell (**A**), testicular homogenate (**B**), and HEK-293 cell (**C**) radioreceptor assay systems. The insets show the relationship between the RRA/I FSH activity ratio and the elution pH value of the isoforms in the corresponding radioreceptor assay systems.

place radiolabeled human pituitary FSH from the rat than the human FSH receptor. Thus, it seems that less acidic/sialylated isoforms are comparatively more active when tested by this particular heterologous receptor. In this vein, it has recently been shown that human recombinant FSH derived from insect cells (and therefore not sialylated *[31]*) binds with a greater affinity to the rat FSH-R than to the human counterpart *(32)*, thus suggesting that the binding capacity of a given FSH isomer may be strongly influenced by the charge on its oligosaccharides. In fact, we and others have shown that graded desialylation of highly acidic rat FSH and less basic rat LH isoforms enhanced their capacity to bind the receptor and induce a biological response *(16,33)*. The mechanism(s) subserving these marked dif-

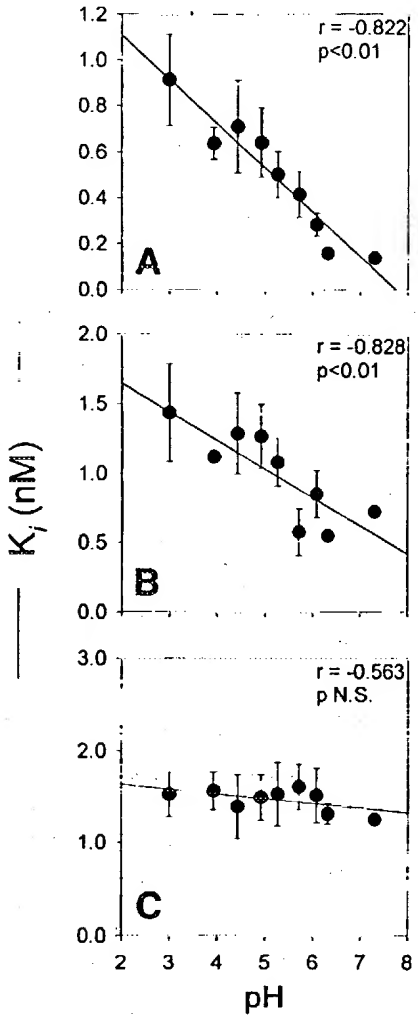

Fig. 3. The relationship between the inhibition constant (K_i) of isoforms I–IX and their corresponding elution pH value.

ferences in binding between the rat and the human receptors is poorly understood; however, one interesting possibility may be that despite the high structural homology between the rat and the human FSH-R *(34,35)*, the former may resolve more effectively (by some still unknown mechanisms) the absence of a steric hindrance imposed by the terminal sialic acid residues, eventually allowing formation of a more stable complex (with a lower off-rate) with low sialylated variants.

Each FSH isoform was then tested (at dose levels ranging from 28 to 2000 ng LER-907/culture dish) for its capacity to induce a biological response in two different in vitro bioassays, one that measures the amount of estrogen produced by rat granulosa cells in culture and the other that determines the amount of cAMP produced by HEK-293 cells expressing the recombinant human FSH-R. The aromatization-inducing activity of isoform I was not considered in this analysis, because this particular variant, which binds with high affinity to both the rat and the human FSH receptor (*36*, present study), has in fact a weak agonist activity and most of its capacity to produce estrogens are rather due to the high amounts of LH material that coelutes with FSH in the chromatofocusing procedure employed *(36)*. In the rat granulosa cell aromatization assay, the biological to immunological (B/I) FSH ratio of the isoforms ranged from 0.51 ± 0.18 to 1.31 ± 0.05, and there was a significant and direct relationship between the B/I ratio and the pH value at which the isoforms were recovered (r = 0.864, $p < 0.01$), with the less acidic variants exhibiting higher ratios than their more acidic counterparts (Fig. 4A). In contrast to the close relationship between receptor binding and signal transduction in the rat granulosa cell system, both functions were clearly dissociated in the homologous system, in which less acidic/sialylated isoforms yielded higher responses in terms of cAMP production than their more acidic counterparts. The corresponding FSH B/I activity ratios in the HEK-293 cell assay were higher than those yielded by the rat granulosa cell assay and ranged from 0.88 ± 0.1 to 4.3 ± 0.6 (Fig. 4B); furthermore, in the homologous assay system, the B/I ratio and pH of the isoforms were directly related ($r = 0.852, p < 0.01$), with the less acidic isoforms presenting the highest ratios. Correlation analysis between the RBA and in vitro bioactivity of the isoforms confirmed the lack of relationship between receptor binding and signal transduction in the human FSH-R system ($r = 0.275$, p NS), whereas this correlation was statistically significant ($p < 0.05$) for the rat granulosa cell assay system ($r = 0.739$).

Figures 4C and 4D finally show the relationship between the B/RRA activity ratio and the elution pH value of the FSH isoforms as disclosed by the heterologous and homologous assay systems. As expected, considering the different behavior of the FSH charge isoforms in the homologous and heterologous RRA systems, the correlation between these two parameters was relatively high ($r = 0.800, p < 0.01$) as disclosed by the HEK-293 cells but not by the rat granulosa cell system.

Differences in receptor activation due to binding may explain the distinct behavior of each isoform in the heterologous bioassay. However, this mechanism cannot account for the observed variations in signal transduction yielded by the homologous receptor system in which no significant differences in RBA among the various isoforms were observed. On the other hand, it has recently been proposed that expression of truncated or altered FSH-R isoforms, some of which may exhibit dominant negative properties probably due to preferential coupling to the G_i–mediated transducing machinery, may account for the pleiotropic actions of the hormone *(24–26)*. Although expression of these receptor variants (or other factors that may potentially modulate the function of the FSH receptor/G-protein system in the granulosa cell, but not in the HEK-293 cell, which in fact is not a true target cell) may also contribute to the differences in in vitro bioactivity of the FSH isoforms in the naturally expressed heterologous receptor system, it

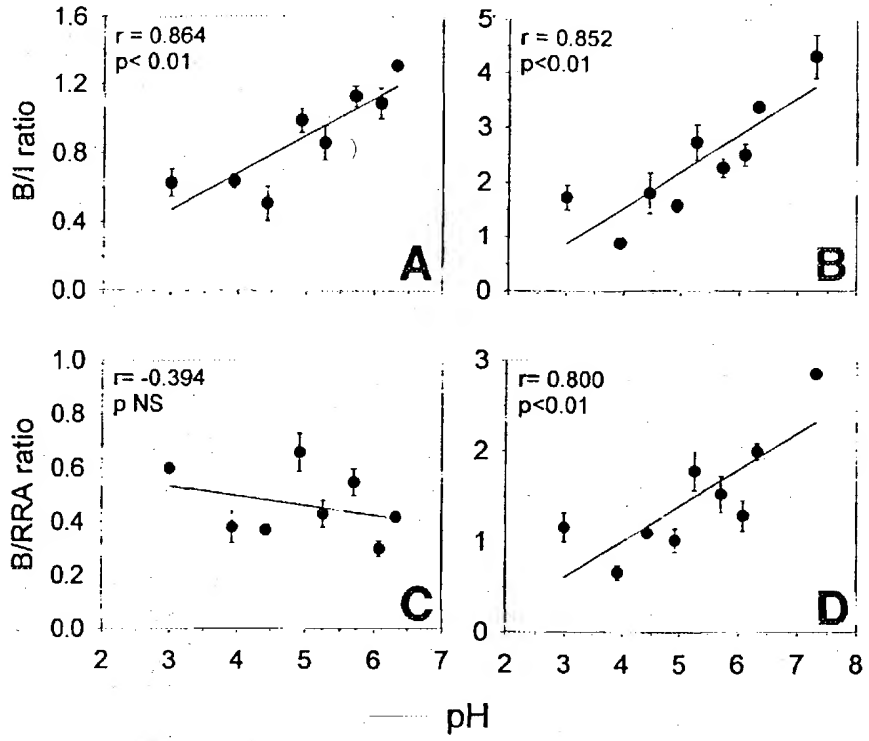

Fig. 4. The relationship between the B/I ratio of the human FSH isoforms as disclosed by the granulosa cell aromatization assay **(A)** and the HEK-293 homologous bioassay **(B)** and their corresponding elution pH value. The lower panels show the correlation between the B/RRA ratio and the elution pH value of the FSH isoforms as disclosed by the granulosa cell **(C)** and the HEK-293 cell **(D)** assay systems.

cannot account for the differences in FSH isoform-induced cAMP production shown by the FSH-R/HEK-293 cell system, which presumptively expresses a unique population of receptors *(37)*. Rather, we hypothesize that the effect of the isoforms on the signal transduction activated by the later receptor system may be related to different abilities of the FSH glycosylation variants to induce and/or stabilize distinct receptor conformations that may permit preferential or different degrees of activation/inhibition of a given signal transduction pathway (G_s- and/or G_i-mediated). In fact, a recent study in Chinese hamster ovary cells and COS-7 cells expressing the human TSH receptor (G_s- and $G_{q/11}$-coupled) has shown that some pituitary TSH glycovariants may differentially activate the two signal transduction pathways to which their cognate receptor is coupled *(38)*. The overall data concurrently indicate that the FSH-R transducer system possesses sufficient versatility to respond in a different manner to glycosylation-dependent diverse FSH signals.

These data further explain the results of previous studies in which short-living, low-sialylated gonadotropin (FSH and hCG) variants behaved as complete agonists of their fully sialylated/long-lived counterparts in in vivo conditions, particularly when an acutely inducible effect was analyzed as the main end point *(21,22)*. Apparently, short-living isoforms may be equally or even more effective than their long-lived analogs owing to their increased ability to activate the receptor and trigger an intracellular signal transduction, compensating and even surpassing the drawback imposed by their relatively short plasma half-life *(21)*. This dual role of the carbohydrate residues, particularly sialic acid, on gonadotropin action is additionally exemplified by recent studies on the closely related glycoprotein TSH, in which an inverse correlation between its B/I ratio and degree of sialylation was observed in various physiological and pathological states *(39)*. Thus, the particular conformation of each glycoprotein hormone isoform plays a key role in determining not only its survival in the circulation, but also the kinetics and effectiveness of its interaction with its cognate receptor and the full expression of a particular in vivo biological effect.

Materials and Methods

Pituitary Extracts

Adult human pituitaries, not selected by sex or age, were collected at autopsies performed after accidental deaths. The bodies were examined no later than 24 h postmortem and were kept at 8°C within 3–4 h after death. The pituitary glands were stored frozen at –70°C until extracts were prepared as described previously *(9)*. Extracts were kept at –70°C until the days of chromatofocusing. The study was approved by the human and animal research ethical committees of the institute.

Chromatofocusing of Pituitary Glycoprotein Extracts

Chromatofocusing of FSH present in pituitary glycoprotein extracts was performed according to the method

described previously *(30,36)*. Briefly, columns of polybuffer exchange resin (PBE-94; Pharmacia Fine Chemicals, Piscataway, NJ) with dimensions 90 × 1.5 cm were constructed and equilibrated with 15 bed volumes of starting buffer (0.025 *M* imidazole-HCl, pH 7.4). Subsequently, a highly concentrated pituitary glycoprotein extract (15–25 mg of immunoreactive FSH), which had been previously equilibrated with the eluent buffer (1:8 dilution of Polybuffer-74 [Pharmacia] in deionized water, pH 4.0) by chromatography in Sephadex G-25 (Pharmacia), was added to the top of the column. Eluent buffer (5 mL) was run before sample application to avoid exposure of the sample proteins to pH extremes. Between 550 and 600 fractions (3 mL each) were collected at a flow rate of 1 mL/4 min at 4°C. The pH of each fraction was measured, and when the pH of the column eluent stabilized at its lowest value, the eluent buffer was then changed to a solution of 1.0 *M* NaCl to recover those proteins bound at the lower limiting pH (salt peak). Each fraction was divided into several aliquots, which were stored frozen at –20°C until measurement of FSH content by RIA. Recoveries of FSH by this method were 79 ± 4% of the total amount applied to the column.

After RIA determination of the amount of FSH contained in 5- to 15-µL aliquots of each fraction collected from three chromatofocusing separations, fractions containing the greatest concentration of each immunoreactive FSH isoform (Fig. 1) were separately pooled, transferred to dialysis membrane tubings (mol-wt cutoff 12,000; Spectrum Medical Industries, Los Angeles, CA), dialyzed at 4°C for 24 h against deionized water, and thereafter against 0.01 *M* ammonium carbonate (pH 7.5), and freeze-dried. Each FSH isoform or pool of neighboring isoforms were redissolved in phosphate- (0.01 *M*) buffered physiological (0.15 *M*/L) saline (PBS; pH 7.4) and kept frozen at –70°C until measurement of FSH content by RIA, RRA, and in vitro bioassays. With the exception of isoform I (*see* Results and Discussion section), negligible amounts of immunoactive prolactin and LH were detected in the final solutions of the different isoform pools added to the RRA tubes and in vitro bioassay culture plates.

RIA of FSH

Purified human FSH (human FSH-I3, NIDDK, Bethesda, MD) was iodinated by the lactoperoxidase-glucose oxidase method *(40)*. Following separation of protein-bound and free ^{125}I by Sephadex G-100 column chromatography, ^{125}I-labeled FSH was further purified by concanavalin A (Con A) chromatography (Pharmacia) as described by Dufau et al. *(41)*. The FSH RIA was performed using reagents provided by the NIDDK; the reference preparation LER-907 (1 mg LER-907 = 53 IU-2nd International Reference Preparation) and the highly purified FSH-I-SIAFP-1 preparation (NIDDK) were employed to construct the standard curves. LER-907, which exhibits a similar degree of charge heterogeneity than crude pituitary extracts as disclosed by chromatofocusing *(42)*, has a potency of 0.004 relative to the FSH-I-SIAFP-1 as disclosed by this RIA system. As with other highly purified pituitary standards *(5)*, the pH distribution profile of FSH-I-SIAFP-1 is limited to a small range where ~70% of the hormone is recovered within a pH of 4.83–4.33 and <25% in a pH ≤ 3.98. The polyclonal antihuman FSH-6 at a final dilution of 1:250,000 was used as antiserum; this antiserum exhibits <0.1% crossreactivity with highly purified human LH and undetectable reactivity with free α-subunit. To minimize the effects of interassay variations as well as to ascertain for parallelism between the unknown samples and the FSH standard, all isoform concentrates prepared were analyzed at multiple dose levels in the same assay run. Inter- and intraassay coefficients of variation were <12 and <8%, respectively, and the sensitivity was 4 ng LER-907/tube.

In Vitro Bioassays of FSH

Rat Granulosa Cell Aromatization Bioassay (GAB)

The capacity of each FSH isoform concentrate to induce aromatization of androgen in vitro was assessed following the method described by Jia and Hsueh *(43)*. Briefly, granulosa cells from diethylstilbestrol- (DES) primed 21-d-old rats were cultured in 16-mm 24-well culture plates at a density of 1×10^5 viable cells in 0.5 mL of McCoy's 5a medium (Life Technologies, Gaithersburg, MD) supplemented with 2 m*M* L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate (Sigma Chemical Co., St. Louis MO), 0.157 m*M* 1-methyl-3-isobutyl-xanthine (MIX; Sigma), 1 µg bovine insulin (Eli Lilly de México, México D.F.), 0.125 µ*M* DES, and 10^{-6} *M* androstenedione (Sigma), and incubated for 72 h at 37°C in 5% CO_2 in the presence of increasing doses of each isoform concentrate or LER-907. At the end of the incubation period, media were collected and stored frozen until quantitation of estradiol by RIA. Inter- and intraassay coefficients of variation were <15 and <8%, respectively. The amount of estradiol produced in vitro was determined by RIA as described elsewhere *(36)*, and the results are expressed as total estrogens produced by the granulosa cells in culture. All samples from a single bioassay were analyzed for estradiol content in the same batch; the mean inter- and intra-assay coefficients of variation were <11 and <6% respectively.

cAMP Production by Human Fetal Cells Expressing the Recombinant Human FSH-R

The human embryonic kidney-derived 293-cell line transfected with the human FSH-R cDNA was a generous gift of Aaron J. W. Hsueh, Stanford, University, Stanford CA. The origin, handling, ligand specificity, and biochemical properties of the recombinant human FSH-R expressed by this cell line have been described elsewhere *(37)*. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies), pH 7.3, supplemented with 5% fetal calf serum, 2 m*M* L-glutamine, 100 mg/mL

geneticin (Life Technologies), 50 U/mL penicillin, and 100 µg/mL streptomycin (Sigma), and grown in 162-cm^2 flasks (Costar, Cambridge, MA). Confluent cells were scraped and plated in 24-well culture plates for 24 h at 37°C in 5% CO_2. Cells (1.5×10^5 cells/culture dish) were then washed and exposed to increasing doses of each isoform concentrate or LER-907 in the presence of 0.125 m*M* MIX for 24 h at 37°C. After incubation, the media and cells were boiled at 90°C for 3 min and stored frozen at –20°C. Total (intra- plus extracellular) cAMP levels were determined by RIA after acetylation of the samples and cAMP standards. The RIA of cAMP was performed employing 2-*O*-monosuccinyl cAMP tyrosylmethyl ester (Sigma) iodinated by the chloramine-T method as the labeling ligand and the CV-27 cAMP antiserum (NIDDK) at a final dilution of 1:150,000. After incubation at 4°C for 24 h, antibody-bound and free cAMP were separated by ethanolic precipitation followed by centrifugation at 1200*g* at 4°C. The sensitivity of the assay was 4 fmol/tube and the inter- and intra-assay coefficients of variation were <12 and <6%, respectively.

Each sample was tested for in vitro bioactivity in triplicate incubations. The relative in vitro biological activity of FSH was calculated considering each dose analyzed in four separate assays and it is expressed as the B/I activity ratio, the ratio of activity in the in vitro bioassay relative to the activity in the immunoassay.

Radioreceptor Assays of FSH

Heterologous RRAs

Rat granulosa cells from DES-primed immature rats were collected by follicular puncture, washed twice in receptor buffer (0.01 *M* phosphate buffer containing 5 m*M* $MgCl_2$, 100 m*M* sucrose, and 0.1% bovine serum albumin, pH 7.5; Sigma), transferred to 12 × 75 mm culture tubes at a density of 2×10^5 cells/tube, and incubated for 18 h at room temperature in the presence of increasing amounts of each isoform concentrate or human FSH-I-SIAFP-1 and ^{125}I-labeled FSH-I-3 (SA, 35 µCi/µg protein). After the incubation period, the reaction was stopped by the addition of 2 mL cold receptor buffer and centrifugation at 1000*g* at 4°C. The supernatant fraction was removed, and the tissue pellets were counted for 10 min in an automatic γ-counter. Nonspecific binding was determined in the presence of 1000-fold excess unlabeled human urinary FSH (Fertinorm HP, Serono de Mexico S.A. de C.V., México D.F.).

Crude membrane preparations (seminiferous tubule homogenate) were obtained from decapsulated testes of 29-d-old Wistar rats as previously described *(9)*. Increasing doses of FSH isoform concentrates or FSH-I-SIAFP-1 and ^{125}I-labeled FSH were incubated with fresh membrane homogenates (diluted in receptor buffer to give a final concentration of 1 mL buffer/g wet wt tissue) at room temperature for 18 h. Reaction was stopped by the addition of cold buffer and centrifugation as described above.

Homologous RRA

HEK-293 cells stably transfected with the human FSH-R were washed twice, resuspended in receptor buffer, and transferred to culture tubes at a density of 2×10^5 cells/tube. The RRA was performed as described above for the rat granulosa cells RRA system.

Saturation analysis of ^{125}I-labeled FSH to FSH-Rs present in the different tissue preparations was performed by adding increasing concentrations of ^{125}I-labeled FSH (1×10^3 to 3.5×10^5, 2.5×10^3 to 6.5×10^5, and 1×10^3 to 4.5×10^5 cpm for the granulosa cell, seminiferous tubule homogenate, and HEK-293 cell receptor assay systems, respectively) to culture tubes containing the FSH-R-bearing tissues and incubated as described above. Dissociation constants (K_d) were calculated as previously described *(44)*.

All RRAs were performed in triplicate incubations. Specific binding ranged from 15 to 19%. Interassay coefficients of variation were 12–15%. The Cheng–Prusoff equation ($K_{d(U)} = IC_{50(U)}/[1 + \{[H]/K_{d(H)}\}]$, where IC_{50} is the concentration of the unlabeled test sample [U] that displaces 50% of bound radiolabeled ligand [H]) was used to calculate the inhibition constants (K_i) of the FSH variants.

Statistical Analysis

Tests for parallelism among the slopes generated by the different FSH preparations (LER-907 and isoform concentrates) in the immunoassays, receptor assays, and bioassays of FSH and calculation of the IC_{50} values were performed following the method of DeLean et al. *(45)*. Correlation analysis was performed to determine the degree of association between two variables (e.g., the elution pH value of the isoforms and their corresponding FSH activity ratios). Probabilities of <0.05 were considered statistically significant.

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